


FORM PTO-1300 (Modified) (REV 11-2000)		U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE		AGENCY'S DOCKET NUMBER 19904-015NATL	
TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371				U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR	
INTERNATIONAL APPLICATION NO PCT/FR99/02964		INTERNATIONAL FILING DATE November 30, 1999 (30/11/99)		PRIORITY DATE CLAIMED December 21, 1998 (21/12/98)	
TITLE OF INVENTION Mammalian Cell Preparations Optionally Transfected With A Gene Coding For An Active Substance Containing Same					
APPLICANT(S) FOR DO/EO/US TIMSIT, Serge				EL 735276209US	
Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information.					
1. <input checked="" type="checkbox"/> This is a FIRST submission of items concerning a filing under 35 U.S.C. 371. 2. <input type="checkbox"/> This is a SECOND or SUBSEQUENT submission of items concerning a filing under 35 U.S.C. 371. 3. <input type="checkbox"/> This is an express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include items (5), (6), (9) and (24) indicated below. 4. <input type="checkbox"/> The US has been elected by the expiration of 19 months from the priority date (Article 31). 5. <input checked="" type="checkbox"/> A copy of the International Application as filed (35 U.S.C. 371 (c) (2)) a. <input checked="" type="checkbox"/> is attached hereto (required only if not communicated by the International Bureau). b. <input checked="" type="checkbox"/> has been communicated by the International Bureau. c. <input type="checkbox"/> is not required, as the application was filed in the United States Receiving Office (RO/US). 6. <input checked="" type="checkbox"/> An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)). a. <input checked="" type="checkbox"/> is attached hereto. b. <input type="checkbox"/> has been previously submitted under 35 U.S.C. 154(d)(4) 7. <input checked="" type="checkbox"/> Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371 (c)(3)) a. <input type="checkbox"/> are attached hereto (required only if not communicated by the International Bureau). b. <input type="checkbox"/> have been communicated by the International Bureau. c. <input type="checkbox"/> have not been made; however, the time limit for making such amendments has NOT expired. d. <input checked="" type="checkbox"/> have not been made and will not be made. 8. <input type="checkbox"/> An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)). 9. <input type="checkbox"/> An oath or declaration of the inventor(s) (35 U.S.C. 371 (c)(4)). 10. <input type="checkbox"/> An English language translation of the annexes of the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371 (c)(5)). 11. <input checked="" type="checkbox"/> A copy of the International Preliminary Examination Report (PCT/IPEA/409). In French 12. <input checked="" type="checkbox"/> A copy of the International Search Report (PCT/ISA/210). Items 13 to 20 below concern document(s) or information included: 13. <input type="checkbox"/> An Information Disclosure Statement under 37 CFR 1.97 and 1.98. 14. <input type="checkbox"/> An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included. 15. <input type="checkbox"/> A FIRST preliminary amendment. 16. <input type="checkbox"/> A SECOND or SUBSEQUENT preliminary amendment. 17. <input type="checkbox"/> A substitute specification. 18. <input type="checkbox"/> A change of power of attorney and/or address letter. 19. <input type="checkbox"/> A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U.S.C. 1.821 - 1.825. 20. <input type="checkbox"/> A second copy of the published international application under 35 U.S.C. 154(d)(4). 21. <input type="checkbox"/> A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4) 22. <input checked="" type="checkbox"/> Certificate of Mailing by Express Mail 23. <input checked="" type="checkbox"/> Other items or information: Limited Recognition; Return Receipt Postcard; Express Mail Label No.: EL 7352769209US Date of Deposit: June 20, 2001					

U.S. APPLICATION NO. (IF KNOWN, S. / CFR		INTERNATIONAL APPLICATION NO. PCT/FR99/02964		ATTORNEY'S DOCKET NUMBER 19904-015NATL	
24. The following fees are submitted.. BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)) : <input type="checkbox"/> Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO \$1000.00 <input checked="" type="checkbox"/> International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO \$860.00 <input type="checkbox"/> International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO \$710.00 <input type="checkbox"/> International preliminary examination fee (37 CFR 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4) \$690.00 <input type="checkbox"/> International preliminary examination fee (37 CFR 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4) \$100.00 ENTER APPROPRIATE BASIC FEE AMOUNT =				CALCULATIONS PTO USE ONLY	
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CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total claims	17 - 20 =	0	x \$18.00	\$0.00	
Independent claims	1 - 3 =	0	x \$80.00	\$0.00	
Multiple Dependent Claims (check if applicable) <input type="checkbox"/>				\$0.00	
TOTAL OF ABOVE CALCULATIONS =				\$990.00	
<input type="checkbox"/> Applicant claims small entity status. (Sec 37 CFR 1.27). The fees indicated above are reduced by 1/2				\$0.00	
SUBTOTAL =				\$990.00	
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Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31) (check if applicable). <input type="checkbox"/>				\$0.00	
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NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.					
SEND ALL CORRESPONDENCE TO:					
<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> Michel Morency Mintz, Levin, Cohn, Ferris, Glovsky and Popeo, PC One Financial Center Boston, MA 02111 </div> <div style="width: 50%;"> <div style="text-align: center;">  SIGNATURE </div> <div style="text-align: center;"> Michel Morency NAME </div> <div style="text-align: center;"> Limited Recognition REGISTRATION NUMBER </div> <div style="text-align: center;"> June 20, 2000 DATE </div> </div> </div>					

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DATE: June 21, 2002

FROM: Barry J. Marenberg

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To:

NAME	COMPANY	BUSINESS #	FAX #
Francine Young			703 746 6713

MESSAGE:

RE: 09/868,663, U.S. National Phase Application Based on PCT/FR99/02964
"Mammalian Cell Preparations Optionally Transfected with a Gene Coding..."
Applicants: Timit and Quinero
Our Docket No. 19904-015 NATL.

Dear Ms. Young:

Attached please find a copy of the transmittal letter for the above-referenced patent application. Please let me know if you have any questions.

We are sending a total of 3 pages, including this cover sheet.

Please call us at 617.348.4966, if you experience any problems.

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PREPARATIONS OF MAMMALIAN CELLS POSSIBLY TRANSFECTED WITH
A GENE CODING FOR AN ACTIVE SUBSTANCE AND FORMULATIONS
CONTAINING SAID PREPARATIONS

The present invention relates to the preparation of genetically modified mammalian cells useful as a model for research and diagnostics or gene therapy, more particularly for the treatment of cerebral nervous system diseases in humans and possibly animals.

Among the new treatments of human disease, gene therapy, i.e. the in vivo correction of the phenotype of a disease using a functional gene as a pharmacological agent, is currently undergoing considerable development.

10 Schematically, a distinction can be made between two types of gene therapy strategy:

- An "in vivo" strategy, where the gene of interest is administered directly into the host's cells.

- An "ex vivo" or cellular gene therapy strategy, consisting of sampling and culturing cells selected as a vector, transferring one or more genes, also referred to as transgenes, in vitro into said cells, and then implanting genetically modified cells.

Cellular gene therapy offers undeniable benefits such as the possibility to be able to verify, *in vitro*, prior to the graft, the effects of introducing and expressing the transgene on the phenotype of the modified
 5 cells, the number of copies of the transgene, its transcription rate, the quantity of protein produced and the biological effect of said protein. The cell population to be grafted may be purified in order to introduce a homogeneous graft in terms of the phenotype
 10 and required protein production.

Among the cells used in cellular gene therapy, in the PCT international patent application published under No. WO93/13807, it was proposed to administer genetically modified non-immortalised endothelial cells intravenously
 15 to express therapeutic products at angiogenic sites.

However, it is important to note that not all endothelial cells are identical. Indeed, adult endothelial cells form a very heterogeneous cell population, not only between organs, but also in the same
 20 organ, between vessels of different sizes. Endothelial heterogeneity is characterised by morphological differences and also by the expression of specific molecular markers for one or more endothelial cell populations. For example, in the central nervous system
 25 CNS, the endothelial cells of cerebral microvessels form, combined with the astrocyte cells of the cerebral parenchyma, the blood-brain barrier BBB.

Therefore, the development of mammalian cell preparations for gene therapy poses a problem in terms of
 30 the homogeneity and characterisation of said cells. An effective solution for this problem consists of immortalising the cells. In this way, immortalised

mammalian cerebral endothelial cell lines or retinal epithelial and endothelial cells possibly comprising a transgene useful for the treatment of neurological diseases including tumours have been described in the prior art. Particular mention may be made of the Applicant's work reported in the PCT international patent applications published under the numbers WO96/11278 and WO97/40139.

The research work conducted by the applicant on the immortalisation of mammalian cells, more particularly cerebral endothelial cells, has enabled it to obtain a significant, homogeneous and perfectly characterised quantity of material to be grafted or injected enabling the implementation of an effective gene therapy method for disease in a patient. In this way, it was demonstrated within the scope of the present invention, that after being injected into the blood compartment irrigating the CNS, genetically modified immortalised cerebral endothelial cells, such as RBE4 cells not expressing a transgene, RBEZ and RBE4/GFP cells expressing a transgene, are capable of surviving and integrating the vascular wall of cerebral micro-vessels, and in the cerebral parenchyma. The demonstration of the interest of this approach required technical expertise in the development of cell preparations and formulations containing said preparations which were injected, and in injection procedures.

Indeed, the Applicant's work relating to the injection of cells in animals enabled it to demonstrate the deleterious effect of the presence of cell aggregates in the injected formulations, such as cerebral vascular accidents or pulmonary embolism. Surprisingly, the

deleterious effect induced by the presence of said cell aggregates during the injection does not seem to have been envisaged to date. However, in the prior art, it was proposed to inject particles conjugated or not with an active agent to carry out a diagnosis or a therapy. Examples include the work conducted on synthetic microspheres of well-defined sizes given below:

- the injection of 75 to 150 micron spheres in heart vessels induces myocardial necrosis (Battler et al., 1993, J. Am. Coll. Cardiol., 22: 2001-2006),
- the injection of 7 micron spheres in pig arteries, at a rate of 105 particles per gram of myocardium, induces no deleterious effect on myocardial tissue (Arras et al., 1998, Nature Biotechnology, 16: 159-162),
- the injection of 48 micron diameter microspheres (900 microspheres) in the internal right carotid induces cerebral infarction in the parieto-temporal cortex, callosum, hippocampus, thalamus and lenticular nucleus (Miyake et al., 1993, Stroke, 24: 415-420).

20 The injection in humans of radiolabelled albumin
microspheres, 15 to 30 microns in size, into the common
or internal carotid arteries to detect infarcted regions
of the brain using cerebral tomo-scintigraphy techniques
has also been described (Verhas et al., 1976, J. Nucl.
25 Med., 17: 170-174), without reporting any deleterious
effect.

In the field of extracorporeal circulation ECC where the particles generated are liable to have a deleterious effect on the body, it has been proposed to use 20 micron filters to reduce the number of potentially deleterious particles by 90% (Loop et al., 1976, Ann. Thorac. Surg., 21: 412-420).

However, as indicated above, the rare work of the prior art relating to the injection of cells, particularly endothelial cells, does not report deleterious effects of injected cell formulations due to the presence of cell aggregates. In this way, the PCT international patent application WO93/13807 describes the intravenous injection of 2×10^6 non-immortalised endothelial cells via the mouse tail vein, and does not mention the observation of deleterious effects related to cell aggregate formation (Ojeifo et al., 1995, Cancer Res., 55: 2240-2244). In the hypothesis in which no deleterious effects were indeed observed, it is probable that the low number of cells injected in a 30 g mouse, of the order of 2×10^6 , i.e. two times less than that carried out within the scope of the present invention on a 300 g rat, does not induce a deleterious effect despite the cell aggregate formation.

Similarly, the authors of the work relating to the intra-arterial (intra-femoral) injection of 1 to 2×10^6 non-immortalised endothelial cells in the lower limb of rats, do not report the observation of deleterious effects and do not suggest the aggregate formation problem (Messina et al., 1992, Proc. Natl. Acad. Sci., 89: 12018-12022). Although this work does not discuss the deleterious effects induced by the injection, it is important to note that the number of cells injected is low, of the order of 50 times less than that carried out within the scope of the present invention. In addition, the target concerned by this work is the vessels of the lower limb, wherein tolerance to ischaemia is greater than in other organs. Moreover, it is indicated that the testers clamped the femoral artery for one hour to enable

a decrease in the blood flow and thus favour the adhesion of the cells to the vascular walls.

Therefore, the aim of the present invention is to offer an effective and simple solution making it possible
5 to prevent the deleterious effects of cell preparation injections and thus develop their application in human medicine.

This aim is achieved by means of a preparation of immortalised mammalian cells possibly transfected with at
10 least one gene coding for an active substance, to be administered systemically in a subject, characterised in that it comprises no aggregate of said cells of a size liable to induce transient or permanent malfunctions in said patient.

15 Preferentially, the immortalised cells are non-tumorigenic.

The preparations according to the invention may thus contain a large number of cells, of the order of 100 to 300,000 cells per microlitre, making it possible to
20 obtain an effective biological effect, for diagnostic or therapeutic purposes, without inducing a deleterious effect liable to induce a transient or permanent decrease in the blood supply of an organ, such as pulmonary embolism, cerebral ischaemic accidents, peripheral
25 ischaemia or even death.

The trials conducted within the scope of the invention made it possible to characterise the size of the aggregates liable to induce deleterious effects during the systemic injection of formulations containing
30 the cells. In this way, advantageously, a preparation according to the invention comprises no cell aggregates of a size greater than approximately 200 microns,

preferentially greater than 50 microns and more preferentially greater than 30 microns.

All cell types, immortalised or not, may be used in the composition of the preparations according to the invention, such as endodermis, epidermis and mesodermis cells, such as cerebral or peripheral endothelial cells and their progenitor, choroid plexus cells, epithelial cells, pigmentary retinal cells, ependymocytes, tancytes, neural progenitor and stem cells, or even embryonic stem cells.

Among these, the invention more particularly relates to mammalian endothelial and epithelial cells, advantageously cerebral or retinal cells.

The immortalisation of the cells may be carried out using any method known to those skilled in the art, such as those described in the PCT patent applications published under the numbers WO96/11278 and WO97/40139. Within the scope of the invention, immortalised are particularly preferred since they offer the advantage of the standardisation of production in large quantities with high quality criteria. The immortalised cells offer a non-tumorigenic characteristic obtained using any method known to those skilled in the art such as those described in the above-mentioned PCT applications.

25 The absence of cell aggregates liable to induce
transient or permanent malfunctions in subjects having
received a preparation according to the invention, may be
obtained using any biological, chemical or physical
treatment preventing aggregate formation or specifically
30 eliminating the aggregate of said cells of a size greater
than approximately 200 microns, preferentially greater
than 50 microns and more preferentially greater than 30

microns. After this treatment, the cells are advantageously suspended in a medium enabling their survival and not favouring their re-aggregation. Such a medium is for example any nutrient medium not favouring aggregation such as calcium and magnesium-free glucose PBS.

A biological treatment of the cells according to the invention consists for example of selecting endothelial cells for specific adhesion criteria or genetically modifying said cells with a nucleic acid sequence expressing an agent preventing aggregate formation or inhibiting the expression of an agent favouring the formation of aggregates of said cells.

Two approaches may thus be implemented:

- 15 - the deletion of sequences coding for adhesion molecules such as: ZO1, ZO2, E-selectin, V.E. Cadherin, ICAM-1, occludin, P-CAM, etc., or
- the introduction of sequences coding for molecules preventing aggregate formation, such as negative dominants of the above-mentioned adhesion molecules or coding for decoy proteins.

A physical treatment of the cells according to the invention consists for example of a filtration or screening. In addition to the exclusion of aggregates, said filtration or screening, offers the advantage of having a cell population of homogeneous size. Said filtration or screening is conducted as follows: the cells are filtered using screening filters, advantageously of 30 microns, and then diluted and dissociated carefully for example by multiple pipetting and the cell suspension is then taken up in a syringe. The filter was immersed beforehand in sterile

physiological saline solution and then disinfected in 100° alcohol, air-dried, immersed again in sterile physiological saline solution. The filter is then placed between the needle and the tip of the syringe containing
5 the cells. The plunger rod is pushed carefully so as to obtain a drop by drop flow of the diluted cells.

However a physical treatment may also consist of "Fluorescent Analysis Cell Sorting" FACS.

A chemical treatment of the cells according to the
10 invention consists for example of trypsinising the cells or subjecting them to the action of another protease.

The cells of the preparations according to the invention may or may not be transfected with one or more genes coding for an active substance which is useful for
15 therapy or diagnostics. Within the scope of the present invention, the term transfection with one or more genes coding for an active substance refers to the transfection of cells with a nucleic acid fragment, such as an expression vector, incorporated in the genome or present
20 in the cytoplasm of the cells, and capable of enabling the expression of polypeptide(s), protein(s) or viral vector directly or indirectly forming an active substance. Examples include immortalised cerebral endothelial cells transfected with a gene coding for an
25 active substance of the formulations described in the international patent application PCT WO96/11278, the disclosure of which is referred to in the present application.

The invention also relates to the use of the above
30 cell preparations for the preparation of a medicinal product intended for the diagnosis or treatment with gene

therapy of a disease in a patient by administering a sufficient quantity of said cells systemically.

Therefore, the invention also relates to a pharmaceutical formulation to be administered
5 systemically in a subject, characterised in that it comprises a cell preparation as described above, combined in said formulation with a pharmaceutically acceptable vehicle enabling the survival of said cells and not favouring their re-aggregation. The term pharmaceutical
10 formulation refers to both therapeutic and diagnostic formulations.

The size of the aggregates which are not liable to induce, during the injection of formulations according to the invention in a patient, transient or permanent
15 malfunctions depends on the administration route. In this way, organ-selective arterial injections go directly to said organ without passing through a filtering organ such as the lung beforehand. Consequently, for intra-arterial administration, the tolerated aggregate size is smaller
20 than for intravenous injection. Indeed, after injection into a vein of the fold of the elbow, the pulmonary filter may act and limit the presence of aggregates in the other organs. However, there is a risk of a deleterious effect with an intravenous injection since
25 the Applicant has observed the death of animals, probably due to pulmonary embolism, with the injection of endothelial cells not having undergone prior filtration.

In addition, an interpretation of the data of the prior art and the experiments carried out by the
30 Application seem to indicate that spheres greater than 40 microns in size are liable to show a deleterious effect on target tissues by the intra-arterial route.

Consequently, if it is considered that a cluster of cells, for example endothelial cells, behaves like a sphere, it is recommended according to the invention to eliminate aggregates greater than 30 microns in size.

5 However, the physical deformability criteria of cells in a micro-vessel are different to those of a synthetic particle, and this parameter must be taken into account during cell treatments, such as for example in filtration, where the use of a 30 micron filter makes it
10 possible to eliminate aggregates greater than 30 microns as much as possible and, consequently, the remaining cells, at least 90%, are isolated cells, wherein the average diameter, for example of an endothelial cell, is 10 microns.

15 Consequently, the invention relates more particularly to:

- firstly, a formulation to be administered by the intra-arterial, advantageously intra-carotid, route, in a patient, characterised in that it comprises a cell
20 preparation comprising no aggregate of said cells greater than 50 microns in size and preferentially greater than 30 microns, and

- secondly, a formulation to be administered by the intravenous route, in a subject, characterised in that it
25 comprises a cell preparation comprising no aggregate of said cells greater than 200 microns in size and preferentially greater than 100 microns.

These two administration routes are to be taken into consideration for the selection of the cells injected
30 into the target organ or tissues. It is indeed recommended to target an organ by injecting the

formulations according to the invention into the artery irrigating the target organ directly.

Conversely, the injection of said formulations by the intravenous route requires having selected or giving
5 the cells specific properties enabling them to target the target organ or tissues. This may consist for example of a selection of endothelial cells showing specific adhesion properties or a genic modification giving it the required properties of the target organ.

10 The intra-arterial injection route, preferentially intra-carotid for applications relating to the CNS, represents a preferential embodiment of the formulations according to the invention. Indeed, although systemic injection appears to be the most suitable since it
15 enables the broadest possible biodistribution, the analysis of this parameter by the Applicant to optimise the gene therapy method implementing the formulations according to the invention led to the preferential selection of the carotid vascular system, which is the
20 blood route closest to the CNS. This system supplies 80% of the cerebral blood flow required in humans for the correct operation of the CNS and is accessible not only in human clinical practice but also for animal testers.

In this way, the Applicant demonstrated within the
25 scope of the present invention that the injection of endothelial cells in the carotid artery is feasible by observing the blood flow rate. The choice of this administration route makes it possible to minimise modifications in the cerebral blood flow as much as
30 possible. Indeed, the flow in the internal carotid artery is never interrupted throughout this procedure. In addition, the analyses conducted on control animals

showed no parenchymatous disorders. In rats, the injection is made in the general carotid circulation and is distributed to the entire region concerned. In humans, it is possible using interventional neuroradiology techniques to inject, using a catheter, smaller vessels, such as the middle cerebral artery, the anterior cerebral artery or the posterior cerebral artery or branches of these arteries and therefore potentially obtain better targeting and a reduced deleterious effect. Naturally, these techniques are invasive, but they are however no more so than an arteriography which requires the same procedures. They are, on the other hand, considerably less invasive than intraventricular or intracerebral injection procedures which could be used to administer a gene therapy product.

Under certain conditions, intra-carotid injection has caused mortality and parenchymatous lesions. The mortality was generally immediate and most frequently associated with respiratory problems. The most plausible explanation is that the injection of cells induced fatal pulmonary embolism. The parenchymatous lesions occurred when the quantities of endothelial cells were high and when the cell suspension was not filtered. This data confirms the concept of the present invention, according to which the cell aggregates are responsible for cerebral parenchymatous lesions and mortality since they are minimised after filtration. The cerebral parenchymatous lesions most probably correspond to cerebral infarctions since they appear in a hypersignal in T2 and are located in the vascular territory of the internal carotid artery. Filtration almost eliminated all these deleterious

effects, in rare cases a dilation of the lateral ventricle was visible on the side of the injection.

As indicated above, the absence of aggregates in the preparations according to the invention provides
5 formulations comprising a higher number of cells than that permitted in the prior art. In this way, the formulations according to the invention comprise of the order of 1000 to 300,000 cells per microlitre of formulation.

10 The formulations according to the invention are particularly useful in the field of gene therapy, but their use may also be envisaged for diagnostic purposes.

Examples of therapeutic applications of the formulations according to the invention include the
15 treatment and/or prevention of degenerative neurological diseases such as Parkinson's, Alzheimer's, Huntingdon's disease, etc., cerebral vascular accidents, cancer, ocular diseases, inflammatory diseases such as rheumatoid arthritis, immunological diseases, arterial or venous
20 vascular malformations.

Among the above therapeutic applications, the invention more particularly relates to a pharmaceutical formulation to be administered systemically, advantageously by the intra-arterial route, in a gene
25 therapy method for a disease of the central nervous system in a subject, characterised in that the cells of the preparation present in said formulation are transfected with at least one gene coding for an active substance in the treatment or prevention of a disease of
30 the nervous system.

The term disease of the CNS refers to the CNS itself, the eye, particularly the retina, and the vessels

forming it or irrigating it. Examples of diseases of the CNS include brain tumours, cerebral infarctions, neuro-degenerative diseases such as those mentioned above, or arterio-venous or simply arterial malformations such as
5 arterial aneurysms or simply venous malformations, ocular diseases, particularly retinal degeneration.

Consequently, the cells of the formulations according to the inventions are transfected with a gene coding for an active substance in the treatment and/or
10 prevention of the above diseases.

The substance coded by the gene with which the cells have been transfected may be directly or indirectly active, i.e. require:

- administration in the subject of a second
15 substance interfering with the first or with the gene coding for said substance, or
- exposure to an energy source, or
- conversion by a substance naturally present in the body,

20 to produce the therapeutic effect.

Particular examples include substances and genes chosen from: growth factors, anti-apoptotic factors, killer genes, antiproteases, immunomodulators, tumour suppressor genes, genes inhibiting the cell cycle, or any
25 other gene or active substance known to those skilled in the art to be useful in the prevention or treatment of diseases of the CNS.

The formulations according to the invention useful for the treatment of a disease of the CNS are for example
30 assayed so as to enable an administration of 1 million to 200 million cells per kilogram of weight of the subject to be treated.

vascular network are at the interface between the blood and the cerebral parenchyma and form the blood-brain barrier characteristic of the central nervous system. It was also demonstrated that they are capable of surviving and being implanted in the central nervous system after an intracerebral graft (Quinonéro et al, Gene therapy, 1997, 4, 111-119).

The work conducted within the scope of the present invention made it possible to demonstrate using three different techniques, bisbenzimidazole staining and reporter genes (beta-galactosidase and GFP), that endothelial cells were capable, firstly of incorporating vessels and, secondly surviving in the parenchyma outside the lumen of vessels. Therefore, these results demonstrate that it is possible to express a transgene in the brain. The therapeutic potential of the formulations according to the invention therefore relates more specifically to diseases of the central nervous system. The diseases more particularly concerned by this approach are brain tumours and cerebral infarctions. Neuro-degenerative diseases may also be concerned, particularly Parkinson's disease, Alzheimer's disease and Huntingdon's disease.

Consequently, the invention more particularly relates to the use of immortalised cerebral endothelial cells, possibly transfected with a gene coding for an active substance for the preparation of a medicinal product intended for the treatment or prevention by gene therapy of a disease of the central nervous system in a subject by administration by the intra-arterial (intra-carotid) route to said subject of a sufficient quantity of said cells to supply said active substance to the central nervous system.

The invention's other advantages and characteristics will be seen more clearly in the following examples relating to the preparation of immortalised cerebral endothelial cells transfected with a gene coding for an active substance and their use in the treatment by gene therapy of a disease of the central nervous system in a patient by administration by the intra-arterial route. These examples refer to the appended figures wherein:

- Figure 1 shows the paranchymatous lesions induced by injections of endothelial cells.
- Figure 2 demonstrates the identification of pre-labelled RBE4 cells in the brain.
- Figure 3 demonstrates the identification of RBEZ cells in the brain after detection of nuclear betagalactosidase by X-Gel.

I - Material and methods

The following three cell lines were used: the RBE4 parental lines and two RBE4-derived lines, the RBEZ line and the RBE4/GFP line. The RBE4 and RBEZ lines are described in the PCT international patent application No. WO96/11278.

The RBE4 line was obtained by the transfection of Lewis rat cerebral endothelial cells in primary culture with an immortalising plasmid containing the ElA sequence of the type 2 adenovirus. The culture conditions for RBE4 cells and RBE4-derived cells have already been described (Durieu-Trautmann et al., Frontiers in CVB, 1993, 331:205-210).

The RBEZ cells were obtained by exposing the RBE4 cells to an MFG-NB non-replicative retroviral vector containing the LacZ gene coding for E. Coli beta-

galactosidase associated with a nuclear localisation sequence (nls) (Lal et al., PNAS, 1994, 91:9695-9699). The RBEZ cells were then selected by FACS (fluorescence-activated cell sorting) using the fluorescent substrate of beta-galactosidase, fluorescein di-beta-galactopyranoside (Lal et al., PNAS, 1994, 91:9695-9699).

The RBE4/GFP line expressing GFP (Green Fluorescent Protein) was obtained after transfecting the RBE4 cells with a construction containing the GFP sequence under the control of the ubiquitin promoter in RBE4 cells.

II - Cell preparation and labelling

The cells in culture were dissociated with trypsin, rinsed several times and suspended in solution at the initial concentration of 300,000 cells per microlitre. The dilution solution used was either glucose PBS (10 mMol) comprising calcium and magnesium, or glucose PBS free of calcium and magnesium. For the injection, different cell concentrations were used. These final cell concentrations ranged from 10,000 cells to 300,000 cells per microlitre. The total injected volume was 500 to 1000 microlitres.

The RBE4 cells in culture were pre-labelled with bisbenzimidazole (Hoechst 33342 Sigma) at a concentration of 7.5 mg/ml for 15 minutes at 37°C. This nuclear colorant is fluorescent blue under ultraviolet light from a fluorescence microscope.

III - Cell filtration.

In some cases, the final solution was filtered using 30 micron screening filters (Polylabo, nylon screen fabric #87404 NY 30 HC) according to the following

protocol: the cells at the initial concentration were taken up using a yellow cone of a P200 pipette to be diluted in the dilution solution. The cells are then diluted and dissociated carefully by multiple pipetting of the preparation using a P1000 pipette and its corresponding blue cone. The cell suspension is then taken up in a 1 ml syringe with a 18 gauge pink needle. The 30 micron filter was immersed beforehand in sterile physiological saline solution and then disinfected in 100° alcohol, air-dried, re-immersed in sterile physiological saline solution. The filter is then placed between the needle and the tip of the syringe containing the cells. The plunger rod is pushed gently to obtain a drop by drop flow of the diluted cells. In the event of difficulties pushing the plunger rod, the filter is replaced. This replacement operation may be carried out up to 2 times per ml. The viability of the cells was measured before and after filtration after Trypan blue staining and reading on a Malassez cell.

20

IV - Intra-carotid injections

Adult male Lewis rats (Iffa-Credo) weighing 300 g on average were anaesthetised with a volatile anaesthetic (Isolflurane) in an oxygen and nitrogen protoxide mixture. A 5 minute induction with 5% isoflurane was followed by a 1% maintenance dose for the operation which generally lasted 30 to 40 minutes. The incision of the cutaneous and subcutaneous tissue was carried out using a monopolar surgical blade. The muscular tissue was then moved to enable correct exposure, on the left, of the common carotid artery, bifurcation of the common carotid artery, the internal and external carotid artery. After

30

dilution buffer of the cell suspension was injected, without the endothelial cells.

V - Tissue imaging study

5 Some rats underwent brain MRI on a 7 Tesla unit (Varian unit) or a spectroscopy. The MRI imaging was carried out with 1 mm joined coronal sections of the entire brain and 500 micron joined coronal sections centred on the anterior brain (telencephal and
10 diencephal, excluding the olfactory bulb). Weighted sequences in T2 were used in the majority of cases. In the rare cases in which the MRI was carried out before 24 hours, a diffusion sequence was added to identify any lesions not visible in T2 sequences. When the brain
15 showed no parenchymatous anomalies in the MRI, a spectroscopy was carried out to compare the right and left spectral profiles in the same animal.

VI- Histological study

20 The animals were sacrificed at different times after injection. In the case of immediate sacrifices, the different organs removed (brain, heart, liver, kidney, eye, spleen, testicle, left carotid artery) were immediately frozen in isopentane cooled with liquid
25 nitrogen. In the case of later sacrifices, the animals had a transcardiac infusion of 100 ml of PBS followed by 500 ml of 4% PFA. The brains removed were post-fixed for 2 to 4 hours in 4% PFA and then cryoprotected in sucrose (20 to 30%) for 48 hours. All the tissue was sectioned
30 with a cryostat either to obtain 30 micron thick slide-mounted sections (all organs) or 40 micron thick floating sections (brain). The floating sections were then

incubated in a solution comprising X-gal for 3 hours 30 for the use of RBEZ cells according to the technique described by Weis et al. (1991). The control tissues were always treated concomitantly.

5

VII- Results

Forty-eight rats were operated on and injected. Nine received a manual injection and 39 an injection using a portable electric motor designed by us to obtain a slow and regular injection. The first operations made it possible to confirm the absence of thrombosis after injection of the common and internal carotid artery.

1) Mortality

Seven rats died prematurely, 5 of which a few minutes after the injection apparently with respiratory problems (n=3), neurological disorders with convulsions (n=1) or with no clear cause (n=1). In all cases, these deaths occurred before the use of the filters. For 6/7 rats, the injected dose was ≥ 50 million of cells. No deaths were identified in the control group (n=10).

2) Deleterious tissular effects.

a) MRI and spectroscopy

In order to study the tissular lesions produced by the intra-carotid injections of genetically modified endothelial cells, we carried out cerebral MRIs and spectroscopy studies. The cerebral MRI represented in figures 1 and 2 appended provides morphological data while the spectroscopy provides chemical data. The spectroscopy was particularly carried out when the MRI

was normal. Twenty MRIs (rats #17, 18, 20, 22, 23, 24, 25, 26, 27, 29, 30 (twice), 31, 32, 33, 34, 35, 37, 38, 39) were carried out and 6 spectroscopies. They were always normal in the control animals (n=3).

5 In the photos in figure 1, 1 mm joined MRI coronal sections, with sequences weighted in T2 are observed. A, B, C: injection of 25 million of non-filtered RBEZ cells; D, E, F: injection of 25 million RBEZ cells after filtration. It is important to note in A, B, C the
10 presence of a cortical and left putaminal hypersignal ipsilateral to the injection which indicates a cerebral infarction; the right and left lateral ventricles (more intense and homogeneous hypersignals than the lesion) are dilated; the lesion also induces a mass effect with
15 displacement of the median line. In D, E, F, the absence of a parenchymatous hypersignal, ventricular dilation and a mass effect are noted.

 In the photos in figure 2, histological cerebral coronal sections enabling the identification of RBE4
20 cells labelled beforehand with bisbenzimidazole and viewed in epifluorescence by an emission in the ultra-violet range, are observed. In A-D, observation of the cerebral parenchyma a few minutes after the intracarotid injection. In F,G, observation 7 days later. The arrows
25 in B and C identify labelled cells in intracerebral micro-vessels. It is important to note in E the presence of labelled cells in the vessels of the choroid plexus. The arrow in F shows a vessel expressing a positive cell. In G, the arrow demonstrates the presence of labelled
30 cells in the choroid plexuses.

 For two rats, the MRI was carried out approximately 15 hours after the injection of the cells and, in these

cases, diffusion sequences were produced in addition to the T2 sequences to ensure that the parenchymatous modifications are displayed well. In the other cases, the MRIs were carried out between 4 and 7 days post-injection. Before the filtration protocol, 13 MRIs were carried out and 7 MRIs after. Before filtration, for the rats injected with 10 million (n=3), no parenchymatous lesions were detected on MRI or spectroscopy; however, a ventricular dilation was visible in 2 of the 3 cases, always on the left side corresponding to the injected carotid artery. Among the rats injected with 25 million (n=4), a ventricular dilation was visible in 3 cases but with no parenchymatous anomaly and one rat showed a parenchymatous hypersignal. In the 3 cases with no anomalies in the MRI, 2 out of 3 had an abnormal spectroscopy. Among the rats injected with 50 million (n=2), both had parenchymatous hypersignal lesions visible in MRI.

After the application of the filtration protocol, the brains of the rats injected with 25 million (n=2) showed no parenchymatous hypersignals, but one had a ventricular dilation. Among the brains of rats injected with 50 million (n=4), only one showed a left cortical hypersignal. However, this rat had been treated previously with mannitol to open the blood-brain barrier. Another, among the 3 remaining rats, had a dilation of the lateral ventricle ipsilateral to the injection.

b) Histology of injected tissue.

The staining of the sections with a Nissl colorant (cresyl violet) did not reveal clear parenchymatous lesions after the injection of filtered cells. However,

staining. However, when the labelling was cytoplasmic, the nuclear labelling was clearly visible, indicating an expression of the endogenous beta-galactosidase (not shown) characteristic of macrophage cells.

5

c) Identification of GFP cells by epifluorescence.

Cells expressing the GFP were visible in the form of a green fluorescent colour 1, 3 and 5 days after the injection of GFP endothelial cells. The green labelling
10 of the GFP was visible both in the cytoplasm and the nucleus of the cell as confirmed by the counter-staining of the nucleus using Bisbenzimidazole. Two types of endothelial cell morphology were visible. Firstly, isolated round cells which appeared to be in endovascular
15 positions and, secondly, elongated cells frequently in groups of 2 within the parenchyma.

CLAIMS

1. Preparation of mammalian cells possibly transfected with at least one gene coding for an active substance, to be administered systemically in a subject, characterised in that it comprises no aggregate of said cells of a size
5 liable to induce transient or permanent malfunctions in said patient.
2. Preparation of mammalian cells according to claim 1, characterised in that it comprises no aggregates of said
10 cells of a size greater than approximately 200 microns, preferentially greater than 50 microns and more preferentially greater than 30 microns.
3. Preparation of mammalian cells according to any of
15 claims 1 to 2, characterised in that said cells are immortalised.
4. Preparation of mammalian cells according to any of
20 claims 1 to 3, characterised in that the cells are non-tumorigenic.
5. Preparation of mammalian cells according to any of
claims 1 to 4, characterised in that said cells are chosen in the group comprising mammalian endothelial
25 cells and epithelial cells.
6. Preparation of mammalian cells according to any of
claims 1 to 5, characterised in that said cells are chosen in the group comprising cerebral and retinal
30 cells.

7. Preparation of mammalian cells according to any of claims 1 to 6, characterised in that said cells have undergone a biological, chemical or physical treatment preventing aggregate formation or specifically
5 eliminating the aggregate of said cells of a size greater than approximately 200 microns, preferentially greater than 50 microns and more preferentially greater than 30 microns, and then suspended in a medium enabling their survival and not favouring their re-aggregation.
- 10 8. Preparation of mammalian cells according to claim 7, characterised in that the biological treatment consists of genetically modifying said cells with a nucleic acid sequence expressing an agent preventing aggregate
15 formation or inhibiting the expression of an agent favouring the formation of aggregates of said cells.
9. Preparation of mammalian cells according to claim 7, characterised in that the physical treatment consists of
20 a filtration or screening.
10. Pharmaceutical formulation to be administered systemically in a subject, characterised in that it comprises a cell preparation according to any of claims 1
25 to 9, combined in said formulation with a pharmaceutically acceptable vehicle enabling the survival of said cells and not favouring their re-aggregation.
11. Formulation to be administered by the intra-arterial,
30 advantageously intra-carotid, route, in a patient, according to claim 10, characterised in that it comprises a cell preparation comprising no aggregate of said cells

greater than 50 microns in size and preferentially greater than 30 microns.

12. Formulation to be administered by the intravenous route, in a subject, according to claim 10, characterised in that it comprises a cell preparation comprising no aggregate of said cells greater than 200 microns in size and preferentially greater than 100 microns.

13. Formulation according to any of claims 10 to 12, characterised in that it comprises of the order of 1000 to 300,000 cells per microlitre of formulation.

14. Pharmaceutical formulation to be administered systemically, advantageously by the intra-arterial route, in a gene therapy method for a disease of the central nervous system in a subject, according to any of claims 10 to 13, characterised in that the cells are transfected with at least one gene coding for an active substance in the treatment or prevention of a disease of the nervous system.

15. Formulation according to claim 14, characterised in the active substance or gene in the treatment or prevention of a disease of the nervous system is chosen from the growth factors, anti-apoptotic factors, killer genes, antiproteases, immunomodulators, tumour suppressor genes, genes inhibiting the cell cycle.

16. Formulation according to any of claims 14 to 15, characterised in that it is assayed so as to enable an administration of 1 million to 200 million immortalised

mammalian cells transfected with at least one gene coding for an active substance per kilogram of weight of the subject to be treated.



DEMANDE INTERNATIONALE PUBLIÉE EN VERTU DU TRAITE DE COOPERATION EN MATIERE DE BREVETS (PCT)

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(54) Title: MAMMALIAN CELL PREPARATIONS OPTIONALLY TRANSFECTED WITH A GENE CODING FOR AN ACTIVE SUBSTANCE CONTAINING SAME (54) Titre: PREPARATIONS DE CELLULES DE MAMMIFERE EVENTUELLEMENT TRANSFECTEES AVEC UN GENE CODANT POUR UNE SUBSTANCE ACTIVE ET LES CONTENANT (57) Abstract <p>The invention concerns a mammalian cell preparation optionally transfected with at least a gene coding for an active substance capable of being administered to a subject by systemic administration. The invention is characterised in that it does not comprise aggregate of said cells having a size likely to cause temporary or permanent dysfunction in the subject. The invention also concerns pharmaceutical compositions comprising said preparation and an acceptable carrier.</p> (57) Abrégé <p>La présente invention a pour objet une préparation de cellules de mammifère éventuellement transfectées avec au moins un gène codant pour une substance active, pour être administrée par voie systémique chez un sujet, caractérisée en ce qu'elle ne comprend pas d'agrégat desdites cellules d'une taille susceptible d'entraîner chez ledit patient des dysfonctionnements transitoires ou permanents. L'invention concerne aussi les compositions pharmaceutiques comprenant une telle préparation et un véhicule acceptable.</p>		

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COMBINED DECLARATION AND POWER OF ATTORNEY FOR PATENT APPLICATION

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am an original, first and joint inventor of the subject matter which is claimed and for which a utility patent is sought on the invention entitled:

MAMMALIAN CELL PREPARATIONS OPTIONALLY TRANSFECTED WITH A GENE CODING FOR AN ACTIVE SUBSTANCE CONTAINING SAME

- ☒ was filed on November 30, 1999 as a PCT application designating the United States, and was assigned PCT/FR99/02964. A United States national phase application was filed on June 20, 2001 and assigned U.S. Serial No. 09/868,663.
- ☐ is attached hereto.

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, §1.56.

- ☐ I hereby claim foreign priority benefits under Title 35, United States Code, §119(a)-(d) or §365(b) of any foreign application(s) for patent or inventor's certificate, or §365(a) of any PCT International application designating at least one country other than the United States listed below and have also identified below any foreign application for patent or inventor's certificate or PCT International application having a filing date before that of the application on which priority is claimed.

[illegible]

Application No. (U.S.S.N.)	Filing Date (dd/mm/yy)	Status (Patented, Pending, Abandoned)

PCT International Application No.	PCT Filing Date (dd/mm/yy)	Status
PCT/FR99/02964	30 Nov. 99	pending

I hereby appoint the following attorneys and/or agents to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith:

- 2 -



DEMANDE INTERNATIONALE PUBLIÉE EN VERTU DU TRAITE DE COOPÉRATION EN MATIÈRE DE BREVETS (PCT)

(51) Classification internationale des brevets ⁷ : A61K 48/00, C12N 5/10, A61P 25/00		A1	(11) Numéro de publication internationale: WO 00/37111
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<p>(54) Title: <u>MAMMALIAN CELL PREPARATIONS OPTIONALLY TRANSFECTED WITH A GENE CODING FOR AN ACTIVE SUBSTANCE CONTAINING SAME</u></p> <p>(54) Titre: PREPARATIONS DE CELLULES DE MAMMIFERE EVENTUELLEMENT TRANSFECTEES AVEC UN GENE CODANT POUR UNE SUBSTANCE ACTIVE ET LES CONTENANT</p> <p>(57) Abstract</p> <p>The invention concerns a mammalian cell preparation optionally transfected with at least a gene coding for an active substance capable of being administered to a subject by systemic administration. The invention is characterised in that it does not comprise aggregate of said cells having a size likely to cause temporary or permanent dysfunction in the subject. The invention also concerns pharmaceutical compositions comprising said preparation and an acceptable carrier.</p> <p>(57) Abrégé</p> <p>La présente invention a pour objet une préparation de cellules de mammifère éventuellement transfectées avec au moins un gène codant pour une substance active, pour être administrée par voie systémique chez un sujet, caractérisée en ce qu'elle ne comprend pas d'agrégat desdites cellules d'une taille susceptible d'entraîner chez ledit patient des dysfonctionnements transitoires ou permanents. L'invention concerne aussi les compositions pharmaceutiques comprenant une telle préparation et un véhicule acceptable.</p>			

all of MINTZ, LEVIN, COHN, FERRIS, GLOVSKY AND POPEO PC, One Financial Center, Boston, Massachusetts 02111, as Applicant's attorneys with full power of substitution and revocation to take any and all action necessary with regard to the above-identified patent.

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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or patent issued thereon.



Inventor's Signature

27/09/01

Date

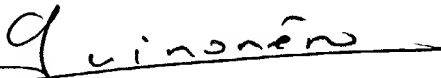
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